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Sexual Reproduction and Fine Structure of Auxospore in *Caloneis linearis* (*Bacillariophyceae*)

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Sexual reproductive processes and the fine structure of auxospores in *Caloneis linearis*, a marine raphid diatom, are described in detail. The auxosporulation of the species is of type IC (Geitler's classification); each of two paired cells produces two isogametes. Two zygotes (young auxospores) are formed with thin copulation mucilage in between the gametangial thecae. The auxospore expands to a cylindrical cell and forms weakly silicified envelopes, called incunabula and perizonium. The features of the auxospore in *C. linearis* are as follows. 1) Small round scales fuse to each other and construct thin layers, wrap both poles of the auxospore forming incunabular caps. 2) The perizonium comprise transverse and longitudinal bands. 3) A series of transverse perizonial bands which wrap the trunk body of the auxospore have open ends and consist of the primary and secondary bands. 4) A series of longitudinal perizonial bands comprises a wide central band with two lateral bands at each side, and are positioned beneath the suture of the transverse perizonial series. 5) The initial epivalve has irregular raphe systems in contrast to the normal valve. Variations in auxosporulation types, morphological differences of incunabula, perizonial bands and initial valves between *C. linearis* and other species of *Caloneis* and *Pinnularia* are discussed.

Key words: Auxospore morphology, *Bacillariophyceae*, *Caloneis linearis*, *Pinnularia*, sexual reproduction.

The raphid diatom genus *Caloneis* Cleve was originally established as a subgroup of the genus *Navicula* (Cleve and Grove 1891) and afterward upgraded to the rank of genus (Cleve 1894). More than 100 taxa (including infra-specific categories) have been described by number of researchers (cf. VanLandingham 1968). Although there has been a long-standing debate on the taxonomic relationship between *Caloneis* and *Pinnularia* Ehrenberg, information solely based on the morphology of the vegetative cells was insufficient to reach a definitive conclusion (e.g. Round et al. 1990, Krammer 2000, Mann 2001). Recently Bruder et

al. (2008) reported that *Caloneis* and *Pinnularia* were indistinguishable in molecular phylogeny: they formed a monophyletic clade but species in the two genera were mixed together. Additional evidence is, therefore, required to further elucidate their taxonomic relationship.

For several years, reproductive characteristics have also become recognized as important features to reveal the evolutionary trends in diatoms (e.g., Medlin and Kaczmarska 2004, Sims et al. 2006). Most diatoms undergo sexual reproduction when their cell size is sufficiently small to enter mitotic divisions. After sexual reproduction, a zygote (young auxospore) expands and an initial cell is formed within it. Much research has been reported in a variety of pennate diatom groups, with special focus on auxospore formation (e.g., von Stosch 1962, 1982, Mann 1982, 1984, Cohn et al. 1989, Mayama 1992, Kaczmarska et al., 2000, Chepurnov et al. 2002, Nagumo 2003, Sato et al. 2004, 2008a, 2008b, Trobajo et al. 2006, Poulíčková 2008, Mann and Poulíčková 2009). Several studies have also reported on the auxospores in *Pinnularia* (Pfitzer 1871, Schmidt 1876, Hashizume 1978, 1985, Suzuki and Mayama 1995, Poulíčková et al. 2007, Poulíčková and Mann 2008). In *Caloneis*, however, there have been only three works on auxosporulation (Geitler 1958, 1973, Mann 1989). In addition, despite the species of both of *Caloneis* and *Pinnularia* having very wide distributions with respect to salinity gradient, marine species have been less intensively studied. Particularly in marine or brackish *Caloneis* species, not only their sexual reproduction and auxosporulation but even vegetative cell characteristics have largely been neglected so far.

Here we focus on a marine species: *Caloneis linearis* (Grunow) Boyer. It was originally described as *Navicula linearis* by Grunow (1860) from the Mediterranean Sea, subsequently transferred to the variant species of *Caloneis liber* by Cleve (1894) and finally raised to the rank of species by Boyer (1927). This species is common in the coastal area of Japan, and the morphology of its vegetative cells has been investigated by Ishii et al. (2009). The present study is a first description of sexual reproduction details and the fine structure of an auxospore of *Caloneis linearis*.

Materials and Methods

The samples of benthic diatoms examined in this study were obtained on June 2007 from the biofilm on *Cladophora sakaii* (*Chlorophyceae, Chlorophyta*) growing in the intertidal zone of the rocky shore, Banda, Tateyama-shi, Chiba Prefecture in central Japan (34°97′58″N 139°76′96″E). The diatoms were directly transferred (without isolation) to PES medium (Provasoli 1966) in plastic cups, and kept at 20°C under cool-white fluorescent light at 10–30 μmol photons m^{-2} s^{-1} (usually 12:12 h L:D). After two–three days, several cells were then isolated to establish unialgal cultures. Crosses were made by inoculating two compatible clones into a cup with PES to induce sexual reproduction, which mostly took place within a week.

The living cells were observed using light microscopy (Optiphot 2, Nikon). Samples for fluorescence microscopy were fixed with 1.0% glutaraldehyde. Staining was performed with 5 μL per slide of DAPI (4,6-diamino-2phenylindole.2HCl) at a concentration of 1 μg mL^{-1} in phosphate buffer. Preparations were sealed with wax to prevent evaporation. The processes of auxosporulation in the plastic cups were directly examined using an inverted microscopy (CK2, Olympus). Cleaned specimens for LM observation were prepared according to the method of Nagumo (1995) and dried on cover slip, and then mounted with pleurax (Mountmedia, Wako). Several individuals, suitable for examination with electron microscopy, were manually picked up by Pasteur pipette under LM and cleaned of all organic matter by the bleaching method

(Nagumo and Kobayasi 1990). For SEM observation, cleaned specimens were placed on a coverslip and coated with osmium for a few seconds in an osmium coater (Neoc-AN, Meiwafosis). The observation using S-4000 or S-5000 scanning electron microscope (Hitachi) was carried out at an accelerating voltage of 3 or 5kV. Full matured auxospores were observed in cross sections using S-4300 (Hitachi), which were obtained by cutting the cell with focused ion beam system (FIB: FB-2100, Hitachi). In case of TEM, the specimens were placed on a formvar-coated copper grid, and examined using JEOL-2000EX transmission electron microscopy (JEOL) operated at 80kV.

Results

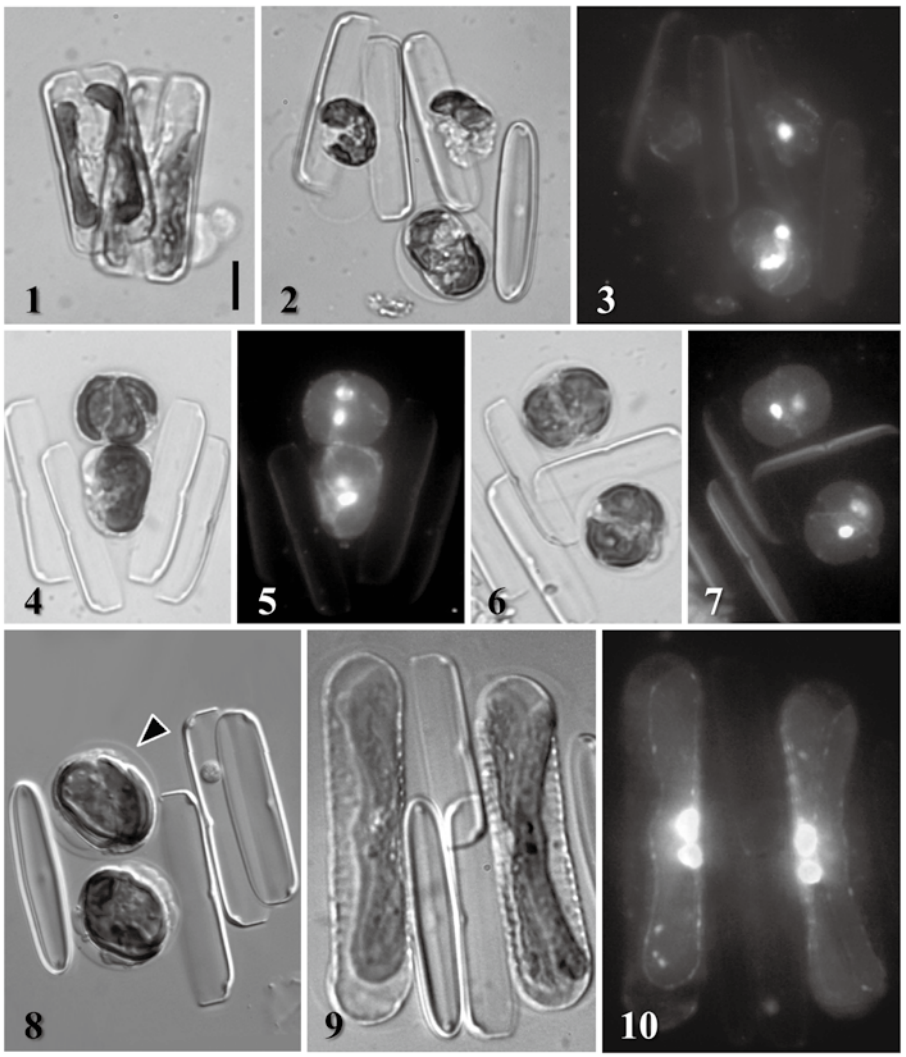
As the vegetative cell morphology of *Caloneis linearis* has been already reported by Ishii et al. (2009), we do not describe the details about it here. The vegetative cells of natural materials ranged 35–70 μm in length. The sexually inducible cells were 35–45 μm in length.

Sexual reproduction and auxospore development

Sexual reproduction and auxosporulation were observed in incubated semi-natural populations and the culture populations in which two monoclonal cultures were mixed. Sexual reproduction did not occur in each monoclonal culture, hence this population of *C. linearis* has the potential of being heterothallic.

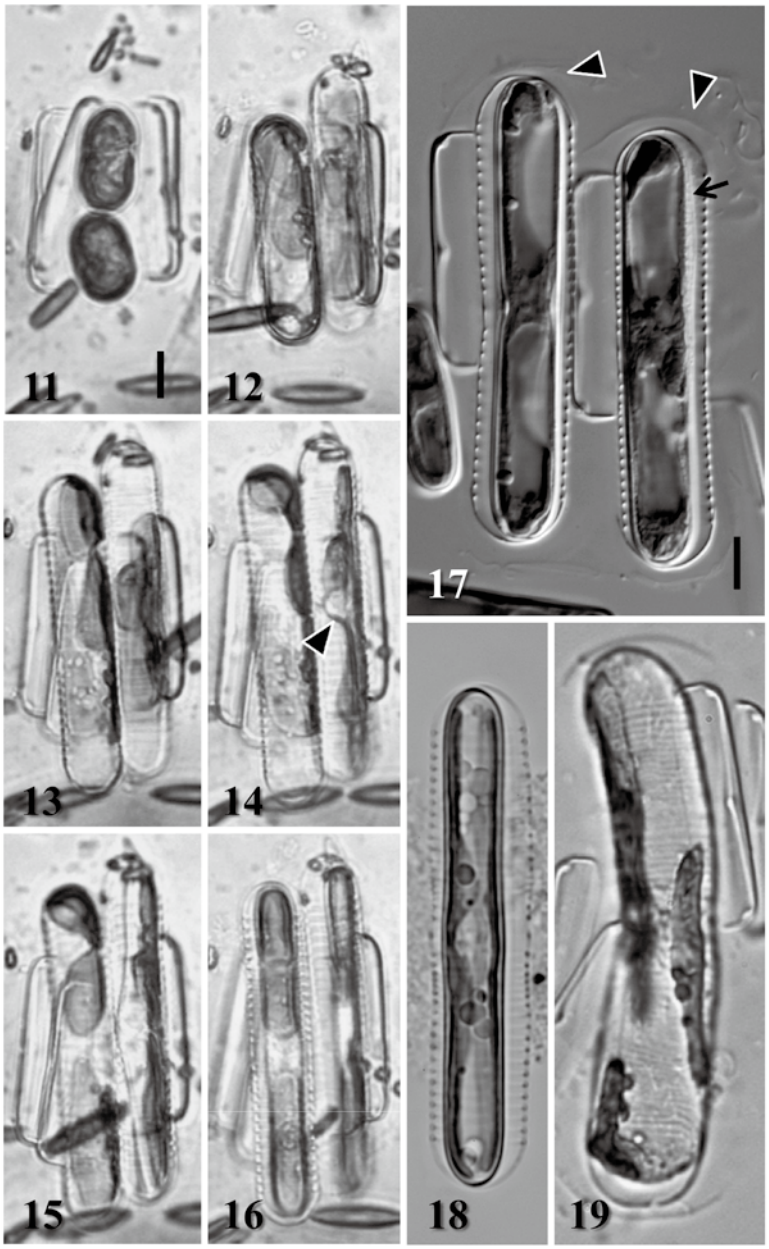
Initially, two compatible cells were in physical contact. When the pairing cells started meiotic division, one cell got upon the other cell in girdle-girdle position to each other. During karyokinesis, the cell cleaved in the normal division plane and two gametes were produced per gametangium (Fig. 1). Each cell contained one nucleus and one plastid. Despite observing many cells, we were unable to document meiosis II. Once each gamete was mature, the gametangial thecae separated. The gametes became rearranged within the thecae and rounding up to the spherical cells (Fig. 2). There was no difference between the two gametes from the same gametangium both in morphology and behaviour. During plasmogamy, four gametes moved to the outside by amoeboid motion and fused to form two zygotes as a result (Figs. 2–8). The zygotes tended to be formed in the interspace of gametangial thecae. The gamete was not surrounded by any mucilaginous envelope (Fig. 2), but the zygote clearly had soft, watery copulation jelly (Fig. 8, arrowhead).

The zygote slightly contracted at first (Figs. 4–7), and shortly afterwards became ellipsoidal and formed the siliceous zygote wall (Fig. 11), which persists as two caps over the elongated auxospore poles during the expansion of the auxospore. The young auxospore contained two plastids and two gametic nuclei which remained unfused until the full expansion of the auxospore (Fig. 10). Subsequently, in most of the observed cells, each auxospore started bipolar expansion parallel to the long axis of gametangial thecae (Figs. 9, 10), though the direction of elongation was not restricted (slanted positions were also sometimes observed). Figs. 11–16 show the processes of the auxospore elongation step by step. The zygote wall was divided in two and a number of silica bands were newly added from the equator of the auxospore to build up the transverse perizonial series (Figs. 9, 11–16). During the expansion of the auxospore, plastids became restricted to one side of the theca, positioned along the long axis and appressed to the wall surface (Figs. 9, 10, 13). In the full elongated auxospore, the gametic nuclei formed one zygotic nucleus, two plastids also migrated to the same side and lay one towards each pole (Fig. 14, right cell; arrowhead show the zygotic nucleus). All protoplasts moved to the side which the nucleus and plastids were already placed (Fig. 15), and the initial epivalve was formed on the same side beneath the transverse perizonial series (Fig. 16). At last the formation of the initial hypovalve occurred in the opposite



Figs. 1–10. Developmental stages of sexual reproduction and auxosporulation in *Caloneis linearis* (LM). Fig. 1. Cytoplasmic divisions of pairing gametangia, two cells are visible in each gamentangium thecae. Figs. 2, 3. One very young zygote (lower cell) containing two gametic nucleus and two plastids, two gametes in gametangium thecae (two upper cells) containing one nuclei (left cell may be unstained) and one plastid (2: Bright field, 3: DAPI stained). Figs. 4–7. Contracted zygotes containing two plastids and two gametic nuclei (4,6: BF, 5,7: DAPI stained). Fig. 8. Two young zygotes, surrounded by thin mucilage envelopes (arrowhead) respectively. Figs. 9, 10. Almost fully expanded auxospores with two appressed plastids and gametic nuclei still not fused (9: BF, 10: DAPI stained). Scale bars: 10 μm.

side (Fig. 17), i.e. the initial cell reached the maturity. Although the existence of longitudinal perizonial bands was confirmed in fully mature auxospores based on many observations, we could not observe the timing of their formation. Thin copulation jellies still remained on the poles of the auxospore (Fig 17, arrowheads), but they subsequently reduced by degrees. The zygote took about ten hours to complete expansion and formation of the initial frustule. There was wide space between the initial cell and transverse perizonial series soon after the completion of the



Figs. 11–19. Auxosporulation and auxospores in *Caloneis linearis* (LM). Figs. 11–16. Auxosporulation stages, series of images of the same configuration over 9 hours. Fig. 11. Young ellipsoidal auxospores. Fig. 12. More than half-expanded auxospores, 4 hours after fig. 11. Fig. 13. Fully expanded auxospores, 6 hours after fig. 11. Fig. 14. One zygotic nucleus is formed at one side (arrowhead) and two plastids lie one towards each pole (right cell), 7 hours after fig. 11. Fig. 15. All protoplast of the auxospore goes to the nuclear side (right cell), 7.5 hours after fig. 11. Fig. 16. Auxospores soon after the formation of the initial epivalve beneath the transverse perizonium, 9 hours after fig. 11. Fig. 17. Fully expanded auxospores, each auxospore is surrounded by mucilage envelope (arrowheads). Left one contains the initial cell in girdle view, right one contains the initial epivalve only (arrow: the longitudinal perizonial band). Fig. 18. Matured auxospores with the initial cell in valve view. Fig. 19. Triplet auxospore containing three chloroplasts. Scale bars: 10 μm.

initial frustule, because the diameter of pervalver axis of the initial cell was very small in relation to the diameter of transverse perizonial series (Fig 18). Immediately afterwards the protoplast began swelling and the space became narrower. The length of completely matured auxospores enclosing the initial cell was 75–85 µm in length. In the culture in which sexualization was vigorous, triplet auxospores were frequently observed, and could get fully expanded but not form the initial frustule (Fig. 19). Finally, the matured initial cell started gliding motion, pushed away the zygote wall of one side and sloughed off its perizonial elements.

Fine structure of the auxospore and the initial valve

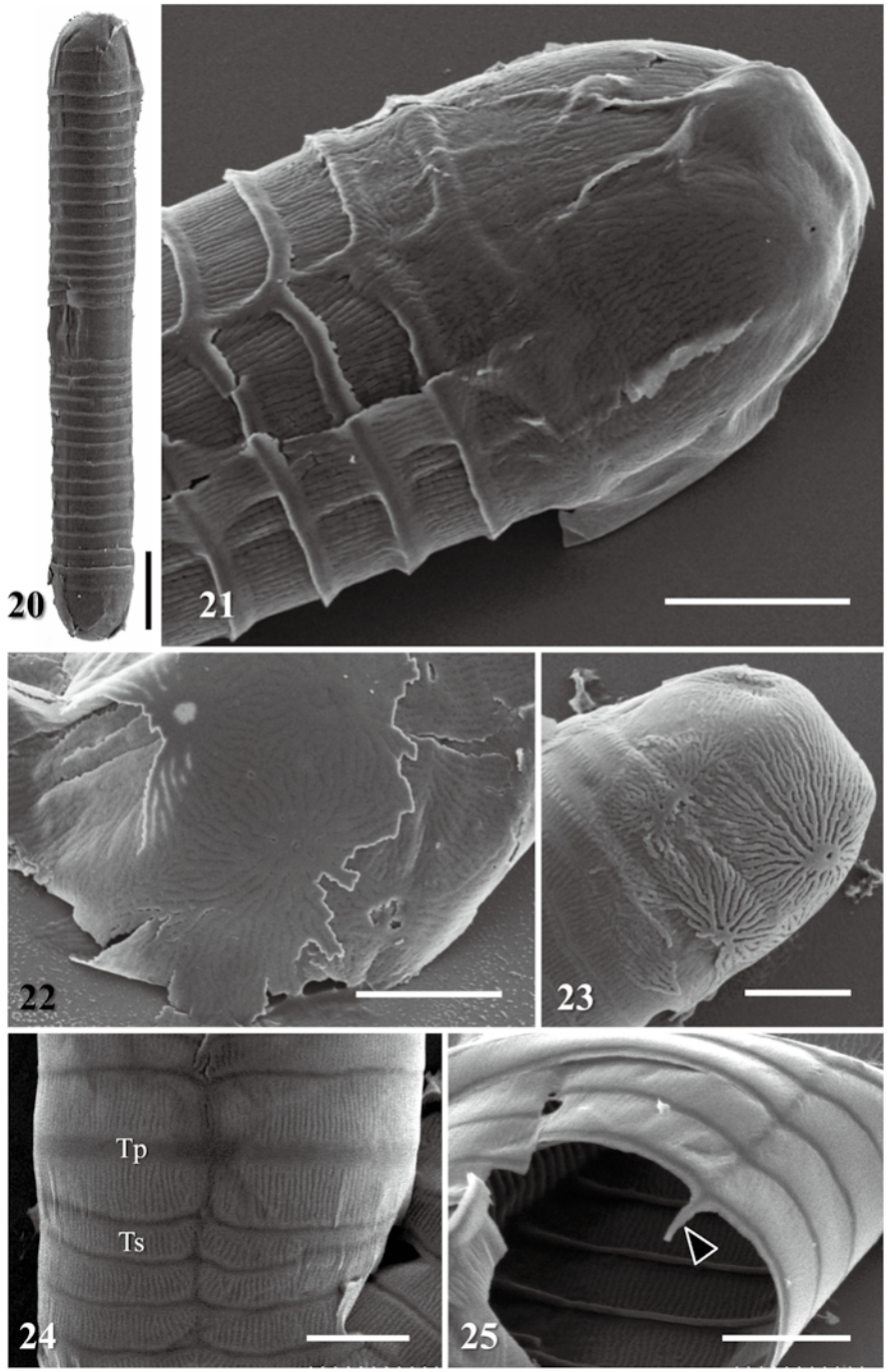
The matured auxospore was cylindrical, surrounded by siliceous components; incunabula and perizonium (Fig. 20).

Each end of the bipolar auxospore was covered by the incunabular cap (Fig. 21), which was formed inside of the copulation mucilage at first. The incunabular cap was formed of few very thin, silicified and delicate layers. Each layer was built out of several small round or ellipsoidal scales, which bore randomly radiated pattern, interfused with each other (Fig. 22). At the bottom layer of the cap, there were partially formed sun-shaped scales, which had several long branches outspread from a small annulus (Fig. 23). The annulus would be the centre of mineralization, and its vestige was also seen in the matured scale (Fig. 22).

Transverse perizonium, placed beneath the incunabula (Fig. 21), comprised a primary band and two series of secondary bands (Fig. 24). The band had sutures running along the midlines (existing on the side of longitudinal perizonium). Both ends of each band were almost completely fused with each other, hence, each band was an evidently incidental ring. These transverse perizonial bands were slightly silicified elements (similar to the incunabular cap). The primary transverse band which occurred initially in between the polarized incunabular caps was a symmetrical cylinder with constriction at the suture line and much wider than the secondary bands. The surface of both sides of the slightly thickened central ridge bore numerous vertical lines seemingly protruding from the ridge. Next to the primary band on each side there were secondary bands series, consisting of about 13–15 pieces each (Fig. 20). Additionally, each band had a ligula-like segment at the fused point (a part of the suture line) (Fig. 25, arrowhead), which curved up towards the primary band and slightly got under the neighboring secondary band (Figs. 21, 24). The ridge of the band (pars media) was placed very close to the equator of the auxospore and distinctly thickened inward from the primary band (Figs. 26, 27). The side of the primary band (pars interior: PI) was very narrow without any pattern or fimbria, but the other side (pars exterior: PE) was wider than PI and striated regularly like the top of the primary band (Fig. 26).

Beneath the transverse perizonial bands, there were five longitudinal bands of three different types. These longitudinal series were most delicate elements, therefore they were almost torn under EM observation. The primary longitudinal band, which existed as one piece beneath the midline of the suture of transverse bands (the opposite side of the initial epivalve) (Fig. 28), was bilaterally symmetrical, with straight margins and consisted of a plain central strip with striated broad sides (Figs. 29, 30). There were two lateral bands on both sides of the primary band : secondary band and tertiary band. Each piece of these bands was overlapped by its more central neighbor. Unfortunately, details of these bands structures could scarcely be examined, because they were almost entirely covered by upper pieces.

The initial frustule was formed at the inside of whole incunabula and perizonial elements following the maturity of the auxospore. The initial epivalve was formed first at the opposite side of the longitudinal perizonial series and the



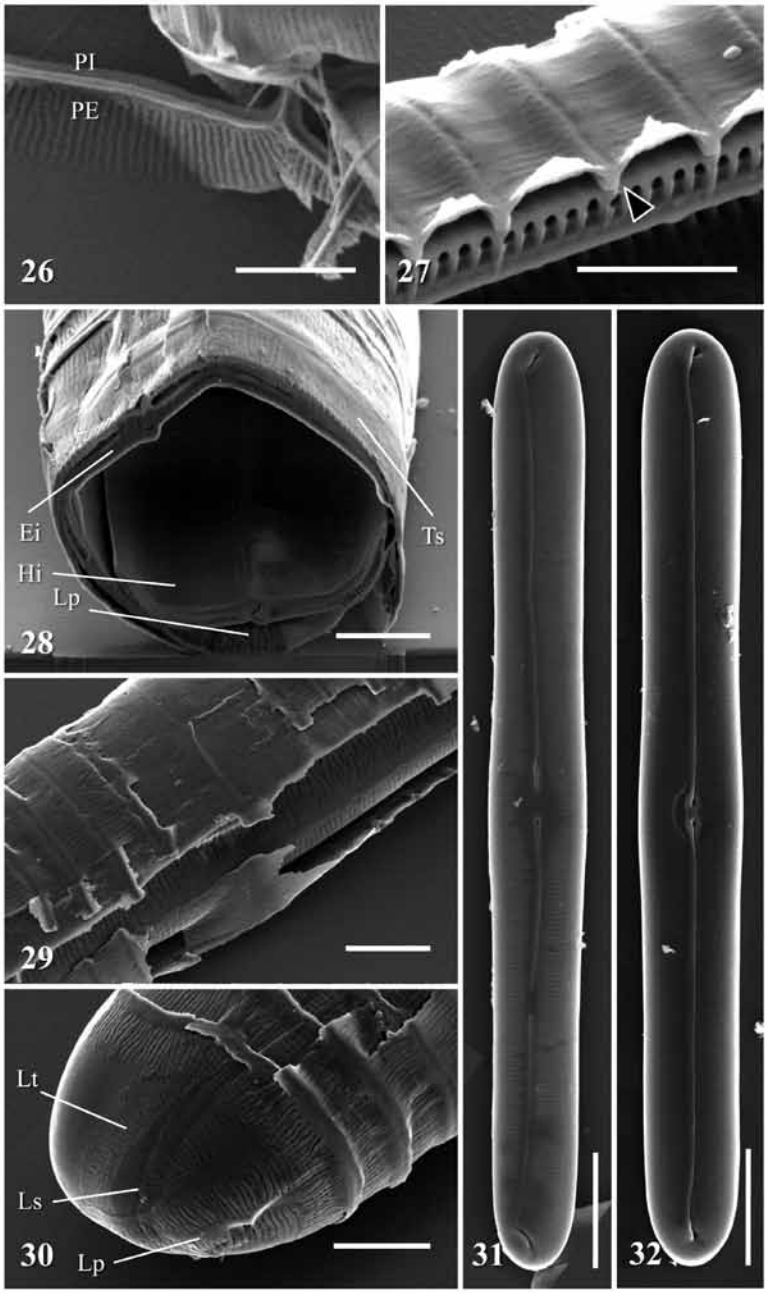
Figs. 20–25. External wall of auxospore in *Caloneis lienaris* (SEM). Fig. 20. Whole image of matured auxospore. Fig. 21. Matured auxospore pole, incunabular cap covers transverse perizonial bands. Fig. 22. Detail of the incunabular cap, which is composed of scale-like components bearing radiate patterns. Fig. 23. Under the whole incunabular cap, developing scales locate immediately above transverse perizonium and initial valves. Fig. 24. Primary transverse band (Tp) and secondary transverse bands (Ts). Fig. 25. Ligula-like segment (arrowhead) on the secondary transverse band. Scale bars: 10 μm (Fig. 20), 5 μm (Fig. 21) and 3 μm (Figs. 22–25).

initial hypovalve occurred subsequently under the longitudinal series. The raphe system of the initial epivalve got irregularly distorted, had discontinuity in spots and the terminal fissures extremely bend to one side (Fig. 31). It was significantly different in structures from normal vegetative valve (Fig. 32) which was introduced by Ishii et al. (2009) in detail. In contrast, the initial hypovalve had obviously straight raphe branches and a semilunar depression at each side of the central nodule as with normal valves.

Discussion

The patterns of auxosporulation in pennate diatoms were classified by Geitler (1973). *Caloneis linearis* belongs to the type IC in his system; each gametangium produces two gametes (two zygotes are formed as a result), which are isogamous both in their morphology and behaviors, and the orientations of auxospores are not fixed to each other or the gametangial thecae because of the copulation envelope being relatively delicate. In the genera *Caloneis* and *Pinnularia*, *Caloneis silicula* (Mann 1989), *C. silicula* var. *truncatella*, *C. alpestris* (Geitler 1973) and *Pinnularia* cf. *gibba* (Poulíčková et al. 2007) were also categorized as type IC. Furthermore, Suzuki and Mayama (1995) described that the gametangia of *P. acidojaponica* (as *P. braunii* var. *amphicephala*) formed two auxospores which are positioned parallel to the long axis of gametangial thecae, although the processes of gametogenesis was then unknown. Hashizume (1985) briefly reported the auxosporulation of *P. viridis*, although his samples seemed to be another *Pinnularia* species to us; each gametangial cell formed two gametes, and as a consequence, two mother cells formed two auxospores (unfortunately there was no description for gamete motion). Thus the researches of Suzuki and Mayama (1995) and Hashizume (1985) suggest that the auxosporulation type of *P. acidojaponica* and *P. viridis sensu* Hasizume (1985) might be also of type IC, or at least of the type I. However, we have also observed type II in other *Pinnularia* species (in preparation), suggesting that the auxosporulation type is not strictly conserved in this group of diatoms. This multiplicity is not surprising given the fact that various types of auxosporulation have also been known in the genus *Nitzschia* Hassall, containing over 900 acceptable morphospecies (Mann 1986) and shows various auxosporulation types (Kaczmarska et al. 2007). In addition, although *C. linearis*, *C. silicula* and *P.* cf. *gibba* display the same type of the auxosporulation, there are some differences in the morphology of copulation envelope among them. Poulíčková et al. (2007) noted that the mucilage envelope around *C. silicula* gametangia is more robust and differentiated than in *P.* cf. *gibba*, possessing a stiff cortical zone. Since *C. linearis* is covered with watery mucilage, it is more similar to *P.* cf. *gibba* than to *C. silicula*.

Incunabula were recently found in *Nitzschia fonticola* by Trobajo et al. (2006) as silicified strips overwrapping the entire surface of the perizonium. Mann and Poulíčková (2009) defined incunabula as components of the auxospore wall external to the perizonium, which partly or completely cover the perizonium as the auxospore expands. The silicified cap structure of the auxospore in *C. linearis* was observed at the outside of the transverse and longitudinal perizonium, hence, it meets the basic definition of incunabula. Although the silicified cap was also reported in *Neidium amphiatum* (Mann 1984, Mann and Chepurnov 2005, Mann and Poulíčková 2009), its structure is appearently robust and consisted of a single hemiellipsoidal part, whereas the cap observed in *C. linearis* seemed to be less robust and clearly built of fused scales. The scales (like in *C. linearis*) are less known, however, they might be a common component in raphid diatoms. Kaczmarska et al. (2000) observed the small circular scales on the auxospore in *Pseudo-nitzschia multiseries*, although the pattern on the surface was ill-defined. Kaczmarska et al.



Figs. 26–32. External wall of auxospore and valves of *Caloneis linearis* (SEM). Fig. 26. Internal view of broken secondary transverse band. Arrowhead indicates the thickened pars media at the fused part. (PI: pars interior, PE: pars exterior). Fig. 27. Longitudinal section of matured auxospore, showing detail of secondary transverse series (arrowhead: pars media). Fig. 28. Cross section of matured auxospore, showing positional relationship between the perizonial elements and initial valves (Ei: initial epivalve, Hi: initial hypovalve). Fig. 29. Primary longitudinal band, exposed by partially missing of transverse series. Fig. 30. Longitudinal series positioning beneath transverse series (Lp: primary longitudinal band, Ls: secondary longitudinal band, Lt: tertiary longitudinal band). Fig. 31. External valve view of initial epivalve with irregular raphe line. Fig. 32. External valve view of normal vegetative valve. Scale bars: 2 μm (Figs. 26, 27), 3 μm (Figs. 28–30) and 10 μm (Figs. 31, 32).

(2007) described almost the same structures in *Nitzschia longissima*. Mann and Poulíčková (2009) remarked that the incunabular caps of *N. amphiatum* examined by LM in Mann (1984) were considered to be a composite structure with several centres of mineralization (though this still has to be confirmed by EM). Although the annulus and radial patterns of the scaly elements, including the caps, are sometimes masked particularly when they are heavily silicified, the silicification of incunabular cap in *C. linearis* is relatively weak so that the radial patterns are clearly visible. The presence of scales has also been known in some araphid diatoms, e.g., *Gephyria media* (Sato et al. 2004), *Grammatophora marina* (Sato et al. 2008a), *Rhabdonema adriaticum*, *R. arcuatum* (von Stosch 1962) and *Tabularia parva* (Sato et al. 2008b). Sato et al. (2004) suggested that the scales are common in the spherical auxospore stage of pennate diatoms, even though there is still no discussion as to whether these scales are homologous.

The incunabula in *Caloneis* and *Pinnularia* is diverse. The incunabular cap of *C. linearis* consisted of siliceous scale-like components. *Caloneis silicula* and *P. viridis* (as *Navicula commutate*) also formed the cap (Mann 1989, Schmidt 1876), but it was uncertain whether the cap is silicified or not because the observations were done only by LM. *P.* cf. *gibba*, *P. acidojaponica* (Poulíčková et al. 2007) and *P. nodosa* (Poulíčková and Mann 2008) bore incunabular strips. Furthermore, we have investigated one *Pinnularia* species bearing both siliceous caps and strips (in preparation). Given the high structural variability of incunabula and various type of auxosporulation detected even within a genus level, these characteristics can also be powerful markers for phylogenetic inference - not only for the estimation for deep branching as implemented by Medlin and Kaczmarska (2004) but also for more recent diversification such as in *Pinnularia* and *Caloneis*. Once these characteristics are obtained from reasonable number of species of *Pinnularia* and *Caloneis* (ideally, together with molecular phylogenetic information), it might become more plausible to refine their taxonomy.

As seen in many raphid diatoms observed so far, the series of transverse perizonium of *C. linearis* comprises a wider primary band flanked by narrower secondary bands on both sides. The primary band is open in *C. linearis,* while closed in *C. silicula* (Mann 1989) and *P.* cf. *gibba* (Poulíčková et al. 2007). Judging from the relatively low accelerating voltage (5 kv) observation of the raphe fissure of initial valve visible through the perizonium (Fig. 20), we conclude that the silicification of perizonial elements in *C. linearis* slightly weaker than that of the other *Caloneis* and *Pinnularia* species. If the primary band of *C. linearis* was more strongly silicified, it would be seen as a closed cylinder with marginal constriction as in *C. silicula* and *P.* cf. *gibba*. In regard to the structure of secondary bands, there is no difference among the species mentioned above. Although the open ends of secondary bands were not directly presented in *P.* cf. *gibba* by any previous observation, Poulíčková et al. (2007) suspected it is due to the small number of auxospore observations and their orientations. As we have recently detected the suture which existed on the opposite side of the initial epivalve in *P. gibba* by SEM, their expectation was confirmed.

The initial epivalve of *C. linearis* had aberrant morphology, especially in a raphe system as in *C. silicula* (Mann 1989). This phenomenon might be natural, assuming that the initial valves are regarded as simply immature valves. The valve face of the initial valve of *C. linearis* is flat as seen in the normal vegetative valves and unlike many raphid diatoms in which the initial valve is usually convex, as it might have been appressed to the inner auxospore wall during its formation. It is worth mentioning that in *C. linearis* there is a space between the initial valve and the transverse perizonium, possibly making the initial valve free from the constraint

from auxospore wall during its morphogenesis.

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石井織葉[a]，出井雅彦[b]，鈴木秀和[a]，南雲　保[c]，田中次郎[a]：**_Caloneis linearis_（クサリケイソウ綱）の有性生殖と増大胞子の微細構造**

海産縦溝珪藻 *Caloneis linearis* の有性生殖と，その結果生ずる増大胞子の微細構造を報告する．本種の増大胞子形成様式は IC 型（Geitler [1973] による分類）である．すなわち，2 つの栄養細胞が対合し，減数分裂により同形配偶子を 2 つずつ形成する．さらに，配偶子嚢殻外で接合が起こり，接合膜に包まれた接合子が 2 つ生じる．接合子は伸長を開始し，インキュナブラおよびペリゾニウムという珪酸質外皮に包まれた円筒形の増大胞子となる．本種の増大胞子は次のような特徴をもつ．(1) インキュナブラは小さな円形鱗片が融合してできた薄層であり，増大胞子の両端を帽子状に包む．(2) ペリゾニウムは横帯と縦帯からなる．(3) 増大胞子を同心円状に包む横帯は 1 枚の第 1 横帯と多数の第 2 横帯からなり，すべて一ヶ所の切れ目をもつ．(4) 横帯の直下（開放端側）に，増大胞子の長軸方向に沿って縦帯が 5 枚配置される．(5) 初生上殻の縦溝は不規則に途切れる．

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